Trp12, a novel Trp related protein from kidney

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Abstract A novel member of the transient receptor potential (Trp) family of ion channels, Trp12, was identified. The Trp12 mRNA is abundantly expressed in mouse kidney and encodes a protein of 871 amino acid residues. Trp12 transfected cells reveal an elevated cytosolic Ca^{2+} and respond with a further increase of cytosolic Ca^{2+} to perfusion with hypoosmotic solutions. The human orthologue of murine Trp12 was localized on a genomic clone derived from human chromosome 12. It is composed of 15 translated exons. The intron placement within that primary structure does not correlate with the previously postulated splice sites in transcripts encoding the stretch-inhibitable channel which shares a high degree of amino acid sequence identity with Trp12 and the vanilloid receptor type 1.

Key words: Transient receptor potential; Osmoregulation; Calcium channel; Cation channel; Capacitative calcium entry

1. Introduction

The family of gene products which are structurally related to the light activated transient receptor potential (Trp) cation channel from *Drosophila melanogaster* [1] is steadily growing [2,3]. The various members of this family share common structural features including the presence of a hydrophobic core region comprising six putative transmembrane segments and a potential pore region between segments 5 and 6. The N-terminal region resides within the cytosol and, typically, comprises several ankyrin-like repeats conserved throughout various members of the Trp family. At least in some members, the presumptive cytosolic C-terminal region harbors domains responsible for interaction with key components of signal transduction processes including calmodulin, NHERF [4] and the inositol 1,4,5-trisphosphate receptor [5,6].

The structural diversity of the various members of the Trp channel family mirrors the broad spectrum of functions which have been associated with these channels. In addition to light activated channels the Trp gene family comprise channels involved in agonist/receptor activated cation influx into cells such as Trp1 to Trp7 [7], osmo- and mechanosensitive ion channels [8,9], channels responsible for pain and heat perception like the vanilloid receptors [10], and epithelial Ca^{2+} channels, ECaC [11] and CaT1 [12], implicated to play a major role in the renal and intestinal reabsorption of Ca^{2+}. The recently cloned Trp8 channel is a member of the latter subfamily and is expressed in prostate, pancreas and placenta but not in the kidney and in the intestine [13]. While Trp8 like ECaC forms highly Ca^{2+} selective cation channels, other members of this Trp subfamily including the vanilloid receptors [10,14], the stretch-inhibitable channel (SIC, [15]) and the growth factor-regulated channel (GRC, [16]) are non-selective cation channels. Trp8, like ECaC, appears to be activated by hyperpolarizing the cell membrane [13,17] whereas the vanilloid receptor (Vr1) is activated by compounds containing a vanilloid moiety including capsaicin [10]. To find a structural and functional link between these two subgroups of channels we looked for a channel structurally related to the Vr1 and ECaC proteins. We addressed this search by a biocomputing approach and identified and cloned the cDNA of a new member of the Trp gene family which shows 32% and 46% sequence identity with the ECaC channel and the Vr1, respectively. Trp12 is abundantly expressed in mouse kidney. The human Trp12 gene encodes a protein of 96% sequence identity with the murine Trp12 protein and resides on human chromosome 12. Transfection of cells with the murine Trp12 cDNA reveals a significant increase of cytosolic Ca^{2+} indicating that the Trp12 protein forms a constitutively active Ca^{2+} entry pathway.

2. Materials and methods

2.1. Cloning of the Trp12 cDNA from murine kidney

Total RNA was obtained from murine kidney as described [18] and poly(A)$^+$ RNA mRNA was isolated using poly(A)$^+$ RNA spin columns (New England Biolabs, Beverly, MA, USA). PCR1, 2, 3 were performed as shown in Fig. 1a using the following primers: 5′-ATG AAG TTC CAG GGC GC-3′ and 5′-CTA CAG TGG GGC ATC GTC C-3′ (PCR1), 5′-GGC TGA GAA GTA CAA ACA G-3′ and 5′-TCA AAG AGG ATG GGC CG-3′ (PCR2) and 5′-GCC GCC ACC ATG GCA GAT CCT GGT GAT G-3′, and 5′-CTA CAG TGG GGC ATC GTC C-3′. The fragments obtained correspond to amino acid residues aa 69–871 (PCR1), aa 1–156 including a part of the 5′ non-translated region (PCR2) and the full length Trp12 coding sequence (aa 1–871) including the stop codon (PCR3). The cDNA fragments were subcloned and five–eight independent clones were sequenced on both strands.

2.2. Northern blot analysis

For Northern blot analysis 5 μg human poly(A)$^+$ RNA from several murine tissues was separated by electrophoresis on 0.8% agarose gels, and thereafter transferred to Hybond N nylon membranes (Amersham Pharmacia Biotech Europe, Freiburg, Germany) as described [18]. The membranes were hybridized in the presence of 50% formamide at 42°C overnight. Alternatively a murine multiple tissue RNA blot (Clontech) was hybridized under the same conditions. The probes used for Northern analysis (Fig. 1a) were a 616 bp *Eco*RI fragment of the EST clone AA139413 corresponding to amino acid residues 335–540 of the Trp12 protein (probe ii), a 516 bp PCR fragment corresponding to amino acids 1–156 and the 5′ non-translated region (probe i) and a 227 bp *Sst*I/*Acc*65I fragment corresponding to

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Fig. 1. Primary structure of mouse Trp12. a: Cloning strategy of the murine Trp12 cDNA and comparison of the deduced Trp12 protein sequence with those of the SIC protein, the type 1 vanilloid receptor (Vr1, [10]) and the olfactory channel (osm-9, [8]). ESTs W53556, AA139413 and AA278027 are derived from a common transcript as shown by PCR (PCR1, 2 3) and Northern blot analysis (see Fig. 3) using Trp12 probes i, ii, iii. The C-terminal Trp12 protein sequence (aa 723–871) shares 96% identical amino acid residues with the corresponding part of the SIC channel (shown in gray); aa 1–379 region of the SIC channel and the transmembrane region of the Vr1 are identical (shown in black). b: Alignment of the Trp12 amino acid sequence with the sequences of *C. elegans* osm-9 [8], rat SIC [15] and the rat vanilloid receptor Vr1 ([10], GenBank accession numbers: AF031408, AB015231, T09054). Amino acid residues are numbered on the right. Residues within Trp12 identical to osm-9, SIC and Vr1 are indicated. c: Hydropathy profile [30] of Trp12: Transmembrane segments S1–S6 were defined as regions with a hydropathy index ≥1.5 using a window of 19 amino acid residues. d: Predicted membrane topology of the Trp12 protein. Putative ankyrin repeats (triangle marker), an *N*-glycosylation site (branched circles) and protein kinase C phosphorylation sites (encircled P) are indicated. e: Phylogenetic tree based on the full length cDNA sequences of Trp12 and Trp12 related mammalian gene products GRC [16], BAA93435, Vrl [14], SIC [15], Vr1 [10], CaT1 [12], CaT2 (AF209196), ECaC [8] and the human ECaC (NM019841), respectively.

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the amino acids 767–842 (probe iii). The probes were labeled by random priming with [α-^{32}P]dCTP. Filters were exposed to X-ray films for 4 days (probe ii) and for 2 weeks (probes i, iii).

2.3. Construction of expression plasmids and transfection of HEK cells

To obtain the recombinant dicistronic expression plasmid pdiTrp12 carrying the entire protein coding regions of Trp12 and the green fluorescent protein (GFP) [19], the 5′- and 3′-untranslated sequences of the Trp12 cDNA were removed, the consensus sequence for initiation of translation in vertebrates [20] was introduced immediately 5′ of the translation initiation codon and the resulting cDNA was subcloned into the pCAGGS vector [21] downstream of the chicken β-actin promoter. The IRES (internal ribosomal entry site) derived from encephalmyocarditis virus [22] followed by the GFP cDNA containing a Ser65Thr mutation [23] was then cloned 3′ to the Trp8b cDNA. The IRES sequence allows the simultaneous translation of Trp12 and GFP from one transcript.

2.4. Measurements of $[Ca^{2+}]_i$ in transiently transfected CHO and HEK cells

Chinese hamster ovary (CHO) and human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Ham's F12 medium including 10% L-glutamine and 10% fetal calf serum was used for CHO cells and Dulbecco's modified Eagle's medium (DMEM including L-glutamine, sodium pyruvate and 1000 mg/l D-glucose) supplemented with 10% fetal calf serum (Life Technologies, Paisley, UK) for HEK cells. Both cell lines were incubated in the presence of 5% CO_2 at 37°C. Cells were plated onto glass coverslips in 35 mm diameter petri dishes 24 h prior to transient transfection with 4 μg DNA in 5 ml of the polycationic SuperFect® (Qiagen, Hilden, Germany). Optical and electrical measurements were performed 24–36 h after transfection.

For experiments, coverslips with cells were transferred to the recording chamber and kept in a modified Ringer's solution containing 145 mM NaCl, 2 mM $CaCl_2$, 2.8 mM KCl, 2 mM $MgCl_2$, 11 mM glucose, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), adjusted to pH 7.2 with NaOH. For experiments with osmotic stimulation of cells the following bath solution was used: 100 mM NaCl, 100 mM mannitol, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7.2 with NaOH. To obtain the standard hypotonic solution osmolarity was adjusted to 230 mosm/l by reducing the mannitol concentration. Additional experiments were performed with the following hypoosmotic stimulus: 10 mM $CaCl_2$, 2.8 mM KCl, 2 mM $MgCl_2$, 11 mM glucose, 10 mM HEPES (pH 7.2 with NaOH) adjusted to 245 mosm/l with NaCl. The hyperosmotic solution contained 195 mM NaCl, 2.8 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM glucose, 10 mM HEPES adjusted to pH 7.2 with NaOH.

Ester loading of intact cells was performed by incubation of cells in the culture medium at 37°C with 2 μM 2-{6-[bis(carboxymethylamino)]-5-[2-[2-[bis(carboxymethyl)-amino]-5-methylphenoxy]ethoxy]-1-benzofuran-2-yl}-1,3-oxazol-5-carbonylacid (fura-2) as an acetoxymethyl ester (fura-2 AM) for 30 min. Cells were then rinsed at least three times with the standard saline solution and recording was started after about 15 min.

The Ca^{2+} indicator dye was excited alternately at 340 or 360 and 380 nm. The UV light was applied by a monochromator equipped with a 75 W xenon lamp (T.I.L.L. Photonics, München, Germany) and directed via an optical fiber into the epifluorescent port of the inverted microscope (Axiovert 135M, Zeiss, Oberkochen, Germany) with an oil immersion objective (Fluar, ×40/1.3, Zeiss). The emitted light intensity was amplified by a photomultiplier (Hamamatsu, Japan) and long-pass filtered at 520 nm. Alternatively, Ca^{2+} imaging experiments were carried out on a different set-up (Axiovert S100 with the same objective) using the IMAGO system from T.I.L.L. Photonics (Martinsried, Germany). Cells were alternately excited at 340 or 360 and 380 nm (10 ms exposures) and images were acquired using the T.I.L.L. Vision software once every 3 s.

Transfected cells were identified by their green fluorescence. GFP was excited at a wavelength of 465 nm and the emitted light was passed through a 520 nm long-pass filter. As control we used non-transfected CHO and HEK cells and mock cells transfected with the pCAGGS/IRES-GFP vector. At least two different transfections were used for each set of experiments. Experiments were carried out at room temperature (20–23°C). Osmolarity was determined with a Wescor 5100C vapor pressure osmometer. Stock solution of 500 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was prepared in demineralized water and buffered with 20 mM HEPES at pH 7.2 (NaOH). All chemicals were obtained from Sigma except for EGTA, as a tetracaesium salt (Fluka) and fura-2 AM (Molecular Probes).

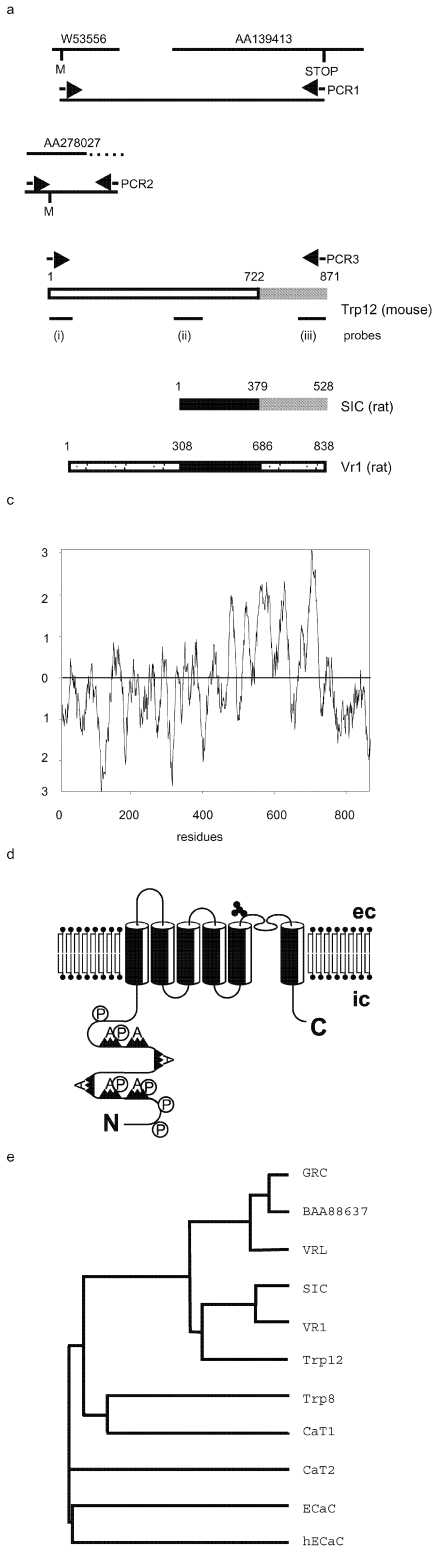
2.5. Miscellaneous methods

Sequences were analyzed using the Heidelberg Unix Sequence Analysis Resources (HUSAR) of the biocomputing unit at the German Cancer Research Centre Heidelberg. The phylogenetic distances of proteins were calculated with the clustal/clustree program [24,25], and the similarity of protein sequences in pairs with the Clustal W algorithm [26]. Analysis was performed with IGOR Pro for Windows (Wavemetrics, Oregon, USA). Throughout, average data are given as means ± S.E.M. for *n* number of cells. Student's *t*-test was used for comparison of means; P values <0.05 were considered to reflect a significant difference. Photographs were scanned and processed using Corel Photo-Paint/Corel Draw and Adobe Photo Shop.

3. Results and discussion

3.1. Cloning strategy to identify the Trp12 gene product

In search of proteins distantly related to the Trp family of ion channels a murine expressed sequence tag (EST AA139413) was identified in the GenBank database. Sequence analysis of this murine EST reveals an open reading frame of 570 amino acid residues, followed by a stop codon, a 3′-untranslated region and a $(poly(A)^+)_{77}$ tail (Fig. 1a). Comparison of the deduced amino acid sequence with the primary structures of members of the Trp family of ion channels reveals similarities to the C-terminal regions of the recently identified subfamily of epithelial Ca^{2+} channels, ECaC, CaT1, and Trp8 [11–13], to the vanilloid receptors [10,14] and to the GRC [16], to SIC [15] and to BAA88637 (published in GenBank database) gene products. Interestingly, a highly homologous sequence was identified on a human genomic clone (GenBank accession number AC007834.25) indicating that this clone harbors the human orthologue of the murine EST. Obviously, the murine gene product, which we tentatively called Trp12, did not cover the complete protein coding region and, consecutively, we used the BAA88637 sequence as



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Trp12  MADPGDGPRAAPGEVAEPPGDESGTSGGEAFPLSSLANLFEGEEGSSSLS  50
osm-9                          MGGGSSRN..KTEPRGEGVKLAFD  22
SIC
Vr1                            MEQRASLDSEESESPPQE.NSCLD  21

Trp12  EVDASRPAGPGDGRPNLRMKFQGAFRKGVPNPIDLLESTLYESSVVPGPK 100
osm-9  P.DEKWSNLYREREKN..HLYKWVAVR....KGG.ELINIYERDGEEGVL  64
SIC
Vr1    PPDRDPNCKPPPVKP...HIFTTRSRTRLFGKGDSEEASPLDCPYEEGGL  68

Trp12  KAPMDSLFDYGTYRHHPSDNKRWRRKVVEKQPQSPKAPAPQPPPILKVFN 150
osm-9  KFAEEKLLTILYD...EGQNP....KLVTYSDYIKWKKGVNVQ....LGL 103
SIC
Vr1    ASCPIITVSSVLTIQRPGDGP....ASVRPSSQDSVSAGEKPP...RLYD 111

Trp12  RPILFDIVSRGSTADLDGLLSFLLTHKKRLTDEFREPSTGKTCLIPKALL 200
osm-9  SEESVDMQ.QSRFKEHYALWKLN....KRGVEGEN.........LIHLLL 139
SIC
Vr1    RRSIFDAVAQSNCQELESLLPFLQRSKKRLTDSEFKDPETGKTCLLKAML 161

Trp12  NLSNGRNDTIPVLLDIAERTGN.MREFINSPFRDIYYRGGTSLHIAIERR 249
osm-9  NRE..Q....QVCYEIARILLKRFPGMANDIYLGDEQFSGSALHLAIVHD 183
SIC
Vr1    NLHNGQNDTIALLLDVARKTDS.LKQFVNASYTDSYYKGGTALHIAIERR 210

Trp12  CKHYVELLVAQGADVHAQARGRFFQPK.........DEGGYFYFGELPLS 290
osm-9  DYETVSLLLNSKADVNARACGNFFLPEDFKLTNKITDYQGYAYYGEYPLA 233
SIC
Vr1    NMTLVTLLVENGADVQAAANGDFFKKT.........KGRPGFYFGELPLS 251

Trp12  LAACTNQPHIVNYLTENPHKKADMRRQDSRGNTVLHALVAIADNTRENTK 340
osm-9  FAACFGNKDIYDLLIQFGANPN...LQDSFSNTILHMCVIN......... 271
SIC
Vr1    LAACTNQLAIVKFLLQNSWQPADISARDSVGNTVLHALVEVADNTVDNTK 301

Trp12  FVTKMYDLLLLKCSRLFPDSNLETVLNNDGLSPLMMAAKTGKIGVFQHII 390
osm-9  YSSSMYSYAVRHWAK..P..ADPHVVNHAGFTPLTLATKLG......... 308
SIC        MYNEILILGAKLHPTLKLEEITNRKGLTPLALAASSGKIGVLAYIL  46
Vr1    FVTSMYNEILILGAKLHPTLKLEEITNRKGLTPLALAASSGKIGVLAYIL 351

Trp12  RREVTDEDTRHLSRKFKDWAYGPVYSSLYDLSSLDTCGE....EVSVLEI 436
osm-9  RKQIFEEMLEIMKVEF..WRFSDMTCSAYPLNTLDTIQPDGSTNYDSALM 356
SIC    QREIHEPECRHLSRKFTEWAYGAVHSSLYDLSCIDTCEK....NSVLEVI  92
Vr1    QREIHEPECRHLSRKFTEWAYGPVHSSLYDLSCIDTCEK....NSVLEVI 397

Trp12  LVYNSKIENRHEMLAVEPINELLRDKWRKFGAVSFYINVVSYLCAMVIFT 486
osm-9  TVINGSTPEHLDMIGSEVIQRLLADKWKAFAQRKLIERLVLLIVQLITLS 406
SIC    AYSSSETPNRHDMLLVEPLNRLLQDKWDRFVKRIFYFNFFVYCLYMIIFT 142
Vr1    AYSSSETPNRHDMLLVEPLNRLLQDKWDRFVKRIFYFNFFVYCLYMIIFT 447

Trp12  LTAYYQPLEGTPPYPYRATV.DYLRLAGEVITLFTGVLFF.FTSIKDLFT 534
osm-9  IVVYIRPTELPRLYMEDPQWDDYIRTACELLTILNCIFFVGYQQLGEIRT 456
SIC    AAAYYRFVEGLPPYKLKNTVGDYFRVTGEILSVSGGVYFF.FRGIQYFLQ 191
Vr1    AAAYYRFVEGLPPYKLKNTVGDYFRVTGEILSVSGGVYFF.FRGIQYFLQ 496

Trp12  KKCPGVNSLFVDGSFQLLYFIYSVLVVVSAALYLAGI.EAYLAVMVFALV 583
osm-9  QGMRGYLRNLKTAPAKAVFCIANLFLLLCIPFRLMKKHEIEEALFVFALP 506
SIC    RR.PSLKSLFVDSYSEILFFVQSLFMLVSVVLYFSQR.KEYVASMVFSLA 239
Vr1    RR.PSLKSLFVDSYSEILFFVQSLFMLVSVVLYFSQR.KEYVASMVFSLA 544

Trp12  LGWMNALYFTRGLKLTGTYSIMIQKILFKDLFRFLLVYLLFMIGYASALV 624
osm-9  GSWIFLLFFARSAKLTGPFVQMIYSMIAGDMIRFAIISAIFLVSFSQVFY 556
SIC    MGWTNMLYYTRGFQQMGIYAVMIEKMILRDLCRFMFVYLVFLFGFSTAVV 289
Vr1    MGWTNMLYYTRGFQQMGIYAVMIEKMILRDLCRFMFVYLVFLFGFSTAVV 594

Trp12  TLLNPCTNMKVCDEDQ.SNCTVPTYPACRDSETFSAFLLDLIFKLTIGMGD 682
osm-9  FVGKDMDAKQKLEDTNPHACRISGY.TIYTYNTFPETFITLFRASMGGYD 605
SIC    TLIEDGKNNSLPMESTPHKCRGSACKPGNSYNSLYSTCLELFKFTIGMGD 339
Vr1    TLIEDGKNNSLPMESTPHKCRGSACKPGNSYNSLYSTCLELFKFTIGMGD 644

Trp12  LEMLSSAKYPVVFILLVTYIILTFVLLLNMLIALMGETVGQVSKESKHI 732
osm-9  YEEFSCANYQALTKTLFVLMFVMPIMMINILIAMMGNTYTTVIAQAEKA 655
SIC    LEFTENYDFKAVFIILLLAYVILTYILLLNMLIALMGETVGQVSKESKHI 389
Vr1    LEFTENYDFKAVFIILLLAYVILTYILLLNMLIALMGETVNKIAQESKNI 694

Trp12  WKLQWATTILDIERSFPVFLRKAFR...SGEMVTVGKS.........SD 782
osm-9  WRQQYAQIVMVLERSVGKERLAASQLEYSIRLDQEGSSGMEVRGLMVIKQ 705
SIC    WKLQWATTILDIERSFPVFLRKAFR...SGEMVTVGKS.........SD 426
Vr1    WKLQRAITILDTEKSFLKCMRKAFR...SGKLLQVGFT.........PD 731

Trp12  GTPDRRWCFRVDEVNWSHWNQNLGIINEDPGKSE.IYQYYGFSHTV.... 814
osm-9  TKKTRARQRKQAIYNWKTIGRKVIHTIDKVSTEQAVLLLHGHDRLDRVYE 755
SIC    GTPDRRWCFRVDEVNWSHWNQNLGIINEDPGKSE.IYQYYGFSHTM.... 471
Vr1    GKDDYRWCFRVDEVNWTTWNTNVGIINEDPSNCEGVKRTLSFSLRS.... 777

Trp12  GRLRRGRWS..TVVERVVELNKNS.............NADEVVVPLDNL 848
osm-9  DHVQPEKVPSRSRTPTRIGTTLNSSKRLKTTTMVVGAAVTNTHEVRTDEA 805
SIC    GRLRRDRWS..SVVERVVELNKNS.............DTDEVVVPLDNL 505
Vr1    GRVSGRNWKNFALVPLLRDASTRDRH............ATQQEEVQLKHY 815

Trp12  GNPNCDGHQQGYAPK.....WRTDDAFL                      871
osm-9  VNSMLLSAPPSLSGEGATMDWQPSITPVEERSESKSQEDRSEASTPNLGI 855
SIC    GNPNCDGHQQGYAPK.....WRAEDAFL                      528
Vr1    TGSLKPEDAEVFKDS.....MVPGEK                        838

osm-9  HRTTPKADSPIRVVEYSRTIRVRAADTIPSIELPNIPTKQTSSTPPHRAV 905

osm-9  SPRLRADMFRRHQQPASFDQSPPLPPTNDKSE                   937
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query to identify an additional murine EST (accession number W53556) with shares high sequence similarity to the N-terminal region of BAA88637 as well as to sequences within the putative human Trp12 genomic clone (AC007834.25). To test whether both murine clones are derived from a common transcript, oligodesoxynucleotide primers were designed covering the nucleotide sequence of AA139413 encoding the very C-terminal amino acid residues and the nucleotide sequence encoding the first methionine in frame of W53556 and adjacent nucleotides (Fig. 1a, PCR1). Using first strand cDNA obtained from transcription of murine kidney poly(A)$^+$ RNA we were able to amplify a DNA fragment of 2409 bp containing the original open reading frame of the two ESTs and, in addition, the central hydrophobic core region comprising six

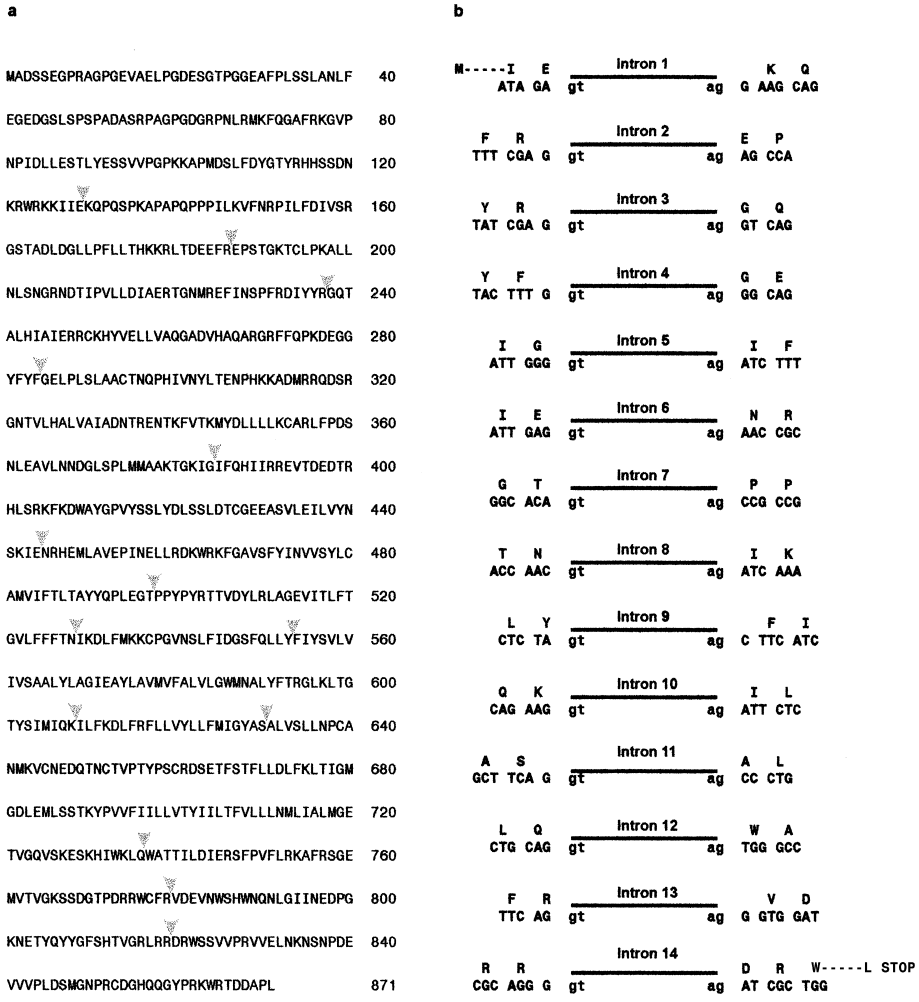

Fig. 2. Structure of the human Trp12 gene. a: Deduced amino acid sequence of the human Trp12 protein predicted from the GenBank clone AC007834 using the TblastN search algorithm and the murine Trp12 protein sequence as query. Predicted splice sites are indicated by (shaded triangle). b: Predicted intron donor and acceptor sites are given in lowercase.

putative transmembrane segments present in all Trp related proteins which have been identified so far. This result indicates that the two murine ESTs are derived from one common transcript. Additional data bank searches yielded several overlapping murine EST clones including EST AA278027 (Fig. 1a). Using, now, oligodesoxynucleotide primers derived from EST AA278027 and EST W53556 we amplified a 516 bp DNA fragment using murine kidney first strand DNA as template (Fig. 1a, PCR2). Sequence analysis of this DNA fragment revealed the codon of the initiation methionine of the Trp12 cDNA within EST AA278027. A methionine residue at corresponding position was identified as initiation methionine within the human genomic Trp12 clone by the GenFinder software. In summary, the full length murine Trp12 cDNA encodes a protein of 871 amino acid residues. This was confirmed by a third PCR using primers derived from the very 5′- and 3′-coding regions of the murine cDNA (Fig. 1a, PCR3). As expected the 2616 bp Trp12 cDNA including the stop codon could be amplified using murine kidney DNA as template.

The analysis of the human genomic clone AC007834.25 revealed a putative protein sharing 96% identical amino acids with the murine Trp12 protein and therefore most probably represents the human orthologue of the Trp12 protein (Fig. 2).

3.2. Primary structure of the Trp12 gene product

Hydropathy analysis reveals a hydrophobic core in the Trp12 protein with six peaks likely to represent membrane spanning helices (S1–S6) and a putative pore region between S5 and S6 (Fig. 1c). The hydrophobic core is flanked by presumptive cytoplasmic domains at the N- and C-termini (Fig. 1d). A similar topology has been proposed for the various members of the Trp family of ion channels from mammals, insects and nematodes. Interestingly, the length of the N-terminal region exceeds considerably the length of the C-terminal part. In addition, six potential phosphorylation sites for protein kinase C and at least six ankyrin-like repeats are localized within the N-terminus (aa 161–185, 191–205, 237–251, 284–298, 309–322, 370–384). A potential N-glycosylation site is localized in the extracellular loop following the S5 segment adjacent to the putative pore region.

Interestingly, the C-terminal region of Trp12 comprising 148 amino acid residues (aa 723–871, Fig. 1b) reveals 96% sequence identity with the corresponding region (aa 380–528) of the rat stretch-inhibitable non-selective channel

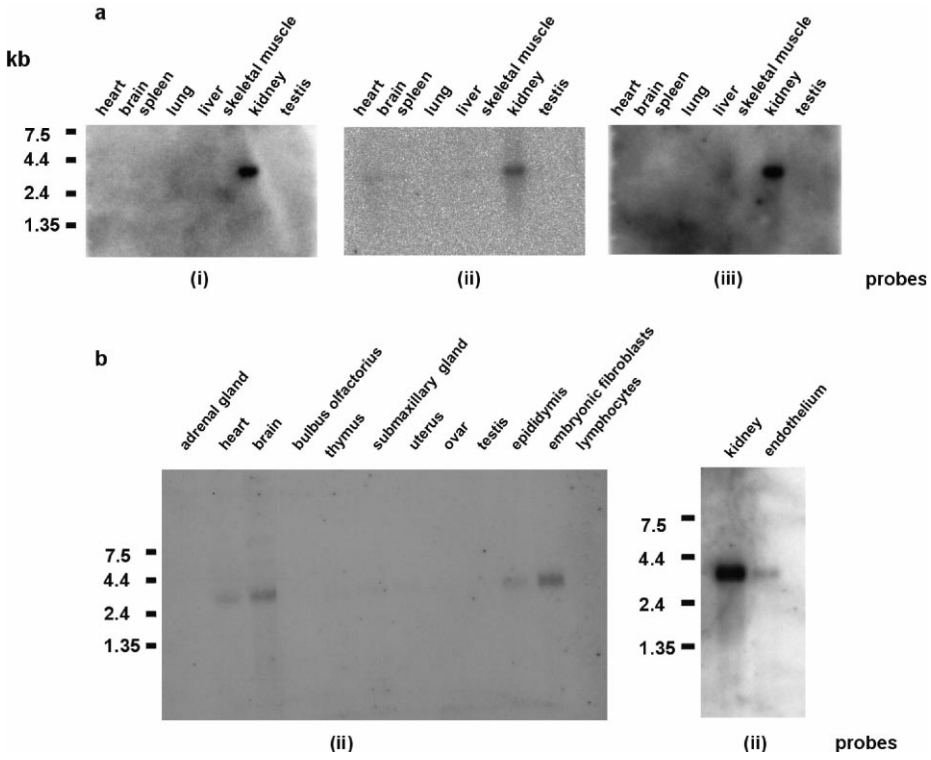

Fig. 3. Expression of Trp12 mRNA in murine tissues. Autoradiogram of RNA blot hybridization analysis using murine Trp12 cDNA probes i, ii and iii (see Fig. 1a) and filter A (a) and probe ii and filters B and C shown in b.

(SIC) indicating that mouse Trp12 and rat SIC may represent splice variants of orthologue mouse and rat genes. Recently, the SIC gene product was suggested to represent a splice variant of the rat Vr1 [27], with which it shares aa 1–379 (Fig. 1a,b). Accordingly, Trp12, SIC and rat Vr1 may represent splice variants of one gene. To prove this, we analyzed the human genomic Trp12 clone (AC007832.25), which is derived from human chromosome 12, and identified 15 exons covering the complete protein coding DNA of the human Trp12 gene product (Fig. 2). The high degree of protein sequence identity (96%) with the murine Trp12 protein (Fig. 2a) allows the unequivocal delineation of the exon boundaries (Fig. 2b). The splice donor and acceptor sites (lower case in Fig. 2b) conform to the sequences for introns [28,29]. The site where the SIC gene product switches from the Trp12-like sequence to the Vr1-like sequence (aa 379/380), is localized within exon 12 of the human Trp12 gene (aa 723/724), making it unlikely that identical splice sites are used to generate Trp12 and SIC.

3.3. Tissue specific expression of Trp12 transcripts

Northern blot analysis using three different cDNA probes demonstrate that Trp12 mRNA is abundantly expressed in murine kidney (Fig. 3). Transcripts of identical size (3.5 kb) are recognized by probes i, ii and iii (Fig. 1a) covering Trp12 DNA encoding the amino-terminal region (i), part of the central core region (ii), and the C-terminal region (iii). The results of these Northern blots confirm the results obtained by PCR (Fig. 1a) that the various parts of the Trp12 cDNA are in fact derived from a common transcript. Less abundant transcripts (3.5 kb) were detected in heart, brain and liver using probe ii (Fig. 3a) whereas no transcripts could be detected by either probe in poly(A)$^+$ RNA from spleen, lung, skeletal muscle and testis. The recognition of transcripts present in heart, brain and liver may be due to cross-hybridization of probe ii, although high stringent hybridization conditions were used. Alternatively these transcripts may arise by alternative splicing and may vary slightly in size from the transcripts in kidney. However, independent Northern blot experiments (Fig. 3b) confirmed expression of 3.5 kb Trp12 transcripts in kidney, heart and brain and revealed additional 3.5 kb Trp12 transcripts in poly(A)$^+$ RNA isolated from epididymis, embryonic fibroblasts and primary cultures of mouse aortic endothelial cells. No transcripts could be detected in adrenal gland, bulbus olfactorius, thymus, submaxillary gland, uterus and ovary.

3.4. $[Ca^{2+}]_i$ is elevated in Trp12 expressing CHO and HEK cells

To characterize the function of the Trp12 protein, CHO and HEK cells were transfected with the murine Trp12 cDNA. We used a dicistronic expression vector which contains the Trp12 cDNA upstream of an internal ribosome entry site (IRES) followed by the cDNA of the green fluorescent protein (GFP). The IRES sequence allows simultaneous translation of Trp12 and GFP from one transcript. Thus transfected cells can be detected unequivocally by their green fluorescence. Transfection of cells with the Trp12 cDNA lead to morphological changes of the cells including rounding of the cell shape and the appearance of blips within the cytosol. Similar changes were not observed in the mock-transfected cells, which express GFP only, nor in the non-transfected control cells.

Using fura-2 fluorescence ratio measurements we studied the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence of

2 mM Ca^{2+} in the bath solution. The $[Ca^{2+}]_i$ was significantly higher in Trp12 expressing CHO (Fig. 4a) and HEK cells (Fig. 4b) compared with mock-transfected cells or control cells. To investigate whether the elevated $[Ca^{2+}]_i$ is due to a constitutively active Ca^{2+} entry pathway, manganese (Mn^{2+}) quench experiments were performed. For this purpose fura-2 loaded HEK cells were excited at the isosbestic wavelength of 360 nm where the emitted light is Ca^{2+} independent but decreases when Mn^{2+} enters the cell and binds to the dye. In the presence of 1 mM $[Mn^{2+}]_o$, seven out of 14 Trp12 expressing cells showed a prominent decrease in fura-2 fluorescence (Fig. 4c, trace a). In addition, the fluorescent ratio decreased (Fig. 4d), indicating an Mn^{2+} block of Ca^{2+} entry. Interestingly, in these cells $[Ca^{2+}]_i$ was significantly higher than in the Trp12 expressing cells which did not reveal any Mn^{2+}-induced decrease in fluorescence. Obviously, Mn^{2+} entry can not be detected in a subset of Trp12 expressing cells under identical conditions (seven out of 14 cells, Fig. 4c, trace b). Similarly, no fluorescent quench was observed in control cells (Fig. 4e) but could be measured after increasing the membrane permeability by the divalent cation ionophore A23187 (Fig. 4f).

Amino acid sequence comparison places the Trp12 protein close to the Vr1 (Fig. 1e), which can be activated by capsaicin. However, capsaicin at 10 μM was without effect on Trp12 expressing HEK cells and control cells ($n = 12$ each, data not shown). A more distantly related member of the subfamily of Trp related gene products is the osm-9 protein (Fig. 1b) which is required for mechanosensation and olfaction in *Caenorhabditis elegans* and which has been implicated to respond to osmotic stimuli [8]. Accordingly, we tested whether hyper- or hypotonic external solutions influence $[Ca^{2+}]_i$ in Trp12 expressing cells. Hyperosmotic stimulation of Trp12 expressing HEK cells did not increase the fluorescent ratio in all cells tested ($n = 4$). In contrast hypotonic stimulation lead to a significant elevation of the fluorescent ratio in a fraction of Trp12 expressing cells, and this elevation was reversible after removing the hypoosmotic solution. Fig. 5a shows representative experiments using two Trp12 expressing CHO cells. Lowering the osmolarity of the bath solution increased the fluorescence ratio in one cell but not in the other. Using the mean value from 19 Trp12 expressing cells this increase is still obvious whereas the control cells ($n = 3$) did not respond to the change in extracellular osmolarity (Fig. 5b). Similar results

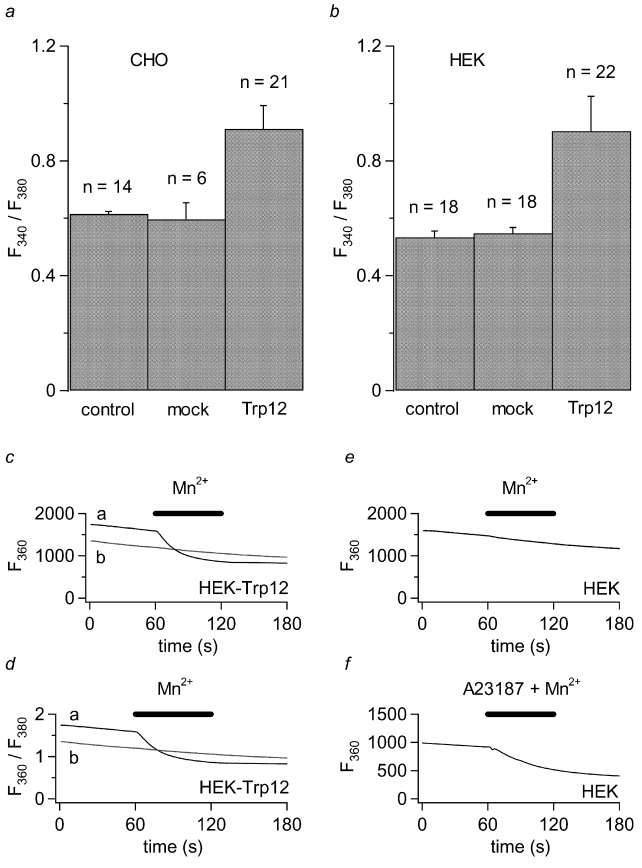

Fig. 4. Elevated $[Ca^{2+}]_i$ in Trp12 expressing cells and Mn^{2+}-induced fluorescent quench. Fluorescent ratios indicate a higher $[Ca^{2+}]_i$ in Trp12 expressing CHO (a) and HEK (b) cells compared to mock-transfected and non-transfected control cells. Application of 1 mM Mn^{2+} in a nominally Ca^{2+} free Ringer solution induced a decrease in fura-2 fluorescence in seven out of 14 Trp12 expressing HEK cells (c, d, trace a) or had no effect in Trp12 cells (c, d, trace b). Control experiments were performed with non-transfected cells by using an identical protocol as in c (e) and after addition of 10 μM A23187 (f, $n = 4$). Fluorescence was elicited at the wavelength indicated. Mean data are shown.

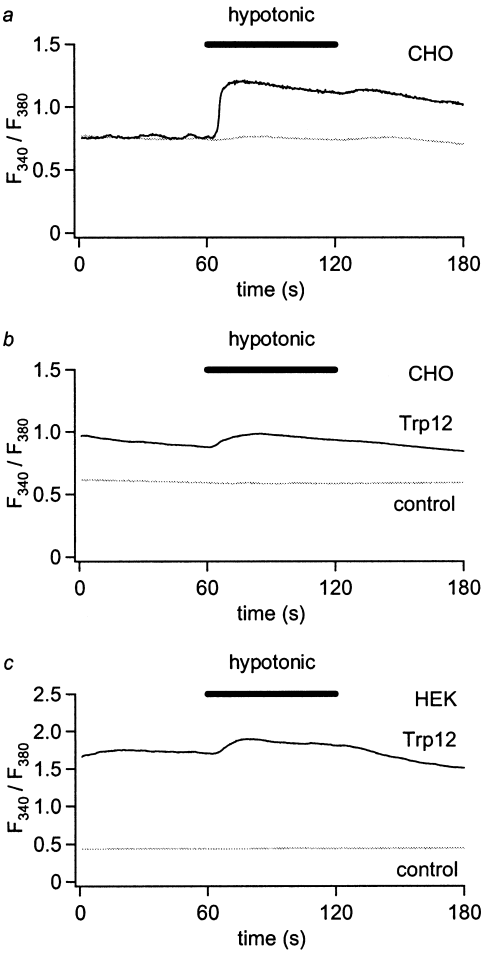

Fig. 5. Effect of hypotonic stimulation on $[Ca^{2+}]_i$. The application of a hypotonic solution (230 mosm/l) increased the fluorescent ratio in one Trp12 expressing CHO cell but not in another (a). Mean data are shown for Trp12 transfected ($n = 19$) and non-transfected CHO (b, $n = 3$) and HEK cells (c, $n = 5$ each). The isotonic extracellular solution was prepared by adding 100 mM mannitol to the application solution.

were obtained using Trp12 expressing HEK ($n = 5$) and HEK control cells ($n = 5$) indicating that the results observed are independent of the cell line used (Fig. 5c). In Mn^{2+} quench experiments 16 out of 34 Trp12 expressing HEK cells showed a decrease of the 360 nm fluorescence signal, which was not observed in control cells ($n = 4$, data not shown). Application of various hypoosmotic stimuli for 1 to 10 min lead to similar results.

Various members of the Trp family including Trp1–Trp7 have been shown to represent Ca^{2+} permeable channels which are activated by depletion of intracellular Ca^{2+} stores or by other mechanisms downstream of G-protein coupled receptors and tyrosine kinase receptor activation [7]. Accordingly the changes of $[Ca^{2+}]_i$ in Trp12 expressing HEK cells and in control cells were monitored after activation of the endogenous muscarinergic receptor and by Ca^{2+} readdition protocols after store depletion. The changes of $[Ca^{2+}]_i$ in HEK Trp12 cells ($n = 7$) and control cells ($n = 12$) were very similar in the presence of 100 μM carbachol. Application of the Ca^{2+}-ATPase inhibitor thapsigargin (2 μM) in the absence of extracellular Ca^{2+} induced a transient increase in $[Ca^{2+}]_i$ due to release of Ca^{2+} from intracellular stores. The subsequent reapplication of 5 mM Ca^{2+} causes an additional increase in $[Ca^{2+}]_i$. Both processes were virtually identical in Trp12 expressing HEK cells ($n = 19$) and control cells ($n = 17$) indicating, that, under the conditions used, Trp12 function is not influenced by the signaling pathway downstream of receptor activation or by thapsigargin-induced Ca^{2+} store depletion. To get insight in the possible involvement of Trp12 expression in the elevation of $[Ca^{2+}]_i$, steady state measurements of $[Ca^{2+}]_i$ were conducted in the absence and in the presence of extracellular Ca^{2+}. In cells, which were first equilibrated for 30 min in a solution containing 10 mM $[Ca^{2+}]_o$ removal of Ca^{2+} leads to a similar decrease of the fluorescence ratio in Trp12 expressing HEK cells ($n = 12$) and control cells ($n = 26$). When HEK cells were equilibrated for 30 min in a Ca^{2+} free solution followed by readdition of Ca^{2+} an increase of the fluorescence ratio was observed. However, this increase was comparable in Trp12 expressing HEK cells ($n = 9$) and controls ($n = 21$).

In summary, changes of $[Ca^{2+}]_i$ attributable to Trp12 expression could only be accomplished by decreasing the osmolarity of the extracellular solution. Approximately 50% of the Trp12 transfected cells respond to hypotonic stimulation whereas none of the non-transfected CHO cells ($n = 3$) and HEK cells ($n = 27$) did. The non-responsiveness of 50% of the Trp12 transfected cells towards hypotonic challenges could be due to low level expression of the Trp12 gene product although no obvious differences in GFP expression levels were noticed between responsive and non-responsive cells. Alternatively, the cells used to express Trp12 may provide at a variable degree endogenous components which could complement the cells' responsiveness towards hypotonic stimuli after Trp12 expression. Further functional studies characterizing the underlying currents may solve this issue.

Finally, Trp12 is high abundantly expressed in the mouse kidney. Likewise, transcripts of Vr1 and of the SIC channel, which is considered to be a short splice variant (528 amino acid residues) of the Vr1 (838 amino acid residues), have been identified in rat kidney. The sizes of these transcripts are 2.3 kb (SIC) and 1.5 kb (Vr1), indicating the expression of proteins, considerably smaller than the Trp12 protein (871 aa). Interestingly, the amino acid sequence of the C-terminal region of Trp12 is very similar to the corresponding region of the SIC. Nevertheless, the Trp12 cDNA probe encoding the C-terminal region recognizes in mouse kidney transcripts of identical size (3.5 kb) as do the Trp12 probes encoding part of the hydrophobic core region and the N-terminus. The size of these transcripts corresponds to the size of the cloned Trp12 cDNA (3.2 kb).

The sequence similarities observed between SIC and Vr1 as well as SIC and Trp12 might indicate that the three proteins arise from one gene by alternative splicing and sharing common exons for Vr1 and SIC, and for Trp12 and SIC. Alternatively, the similarity may be due to the existence of closely related independent genes which may be due to the duplication of a common ancestral gene. Analysis of the human Trp12 gene and delineation of its exon–intron borders (Fig. 2) is a first step to address this issue. Unfortunately, the mouse and human orthologues of rat Vr1 and rat SIC have not been described excluding direct comparison of the DNA sequence of mouse/human Trp12 with those of the mouse/human Vr1 and SIC.

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